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Effects of Pemirolast and Tranilast on Intimal Thickening After Arterial Injury in the Rat

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Summary: We previously reported that tranilast, an antiallergic agent, reduced intimal thickening after endothelial injury in rats. In this study, to verify whether or not antiallergic agents inhibit intimal thickening, we investigated the effect of pemirolast on intimal thickening after endothelial injury and compared its effect with that of tranilast. Administration of two antiallergic agents, pemirolast (0.1, 1, and 10 mg/kg, p.o.) and tranilast (300 mg/kg, p.o., daily), was begun 2 days before endothelial injury and continued until the animals were killed. Endothelial injury in the rat femoral artery was induced by a photochemical reaction between localized irradiation by green light and intravenously administered rose bengal. To evaluate intimal hyperplasia, we measured the cross-sectional area of the intima 21 days after endothelial damage. Pemirolast at doses of 0.1, 1, and 10 mg/kg reduced the intimal area to 2.10 ± 0.33, 1.36 ± 0.19, and 1.35 ± 0.18 ($\times 0.01$ mm^2), respectively, and tranilast showed a tendency to reduce the intimal area, which was 1.86 ± 0.35 × 0.01 mm^2, compared with findings for controls (2.83 ± 0.49 × 0.01 mm^2). In rat A10 vascular smooth-muscle cells, we investigated the effects of antiallergic agents on migration by using a modified Boyden chamber assay and on proliferation by using the bromodeoxyuridine-incorporation assay. Two antiallergic agents inhibited in a concentration-dependent manner both migration and proliferation of smooth muscle cells stimulated by platelet-derived growth factor. These results suggest that antiallergic agents directly inhibit migration of smooth-muscle cells to the intima from the media and proliferation in the intima, and that pemirolast has more potent antihyperplastic action than does tranilast. Antiallergic agents may be effective in preventing restenosis after coronary angioplasty. **Key Words:** Pemirolast—Tranilast—Intimal thickening—Endothelial injury—Migration—Proliferation.

Percutaneous transluminal coronary angioplasty (PTCA) is a well-established therapeutic option in the treatment of patients with arteriosclerotic coronary artery disease. However, this method is limited by the occurrence of restenosis within 6 months after successful angioplasty in ~30–40% of patients (1,2). Several experimental studies strongly support the concept that migration of vascular smooth-muscle cells to the intima from the media and proliferation of smooth-muscle cells in the intima are key events in the restenosis of coronary arteries after PTCA (3,4), but the precise mechanisms of restenosis are not yet fully understood. Although several animal studies of antiplatelet (5,6), anticoagulant (7–9), and antihypertensive agents (10–12) have been reported in which agents inhibited intimal thickening in the balloon-injury model, clinical studies (13–15) have failed to identify an appreciable reduction in the rate of restenosis after PTCA.

We previously reported that tranilast, an antiallergic agent, suppressed intimal thickening after photochemically induced thrombosis in the rat (16). In studies involving human subjects, it was reported that the rate of restenosis after PTCA was reduced markedly in patients administered tranilast compared with controls (17,18). However, the detailed mechanism(s) inhibiting intimal thickening by tranilast has not yet been clarified. It is unclear whether these mechanism(s) are related to antiallergic action(s) (19). Pemirolast, an antiallergic agent, has been in clinical use for treatment of patients with asthma and allergic rhinitis in Japan. The antiallergic actions of pemirolast are more potent than those of tranilast (20,21). Therefore to verify whether antiallergic agents inhibit intimal thickening, we investigated the effect of pemirolast on intimal thickening after endothelial injury and compared its effect with that of tranilast.

MATERIALS AND METHODS

Treatment

9-Methyl-3-(1H-tetrazol-5-yl)-4H-pyrido[1,2-a]pyramidin-4-one potassium salt (pemirolast) and *N*-(3,4-dimethoxycin-

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namoyl) anthranilic acid (tranilast) were suspended in 0.5% carboxymethyl cellulose (CMC). Seventy-five male Wistar rats weighing 170–210 g were purchased from SLC (Hamamatsu, Japan) and were administered pemirolast and tranilast orally once a day at doses of 0.1, 1, 10, and 300 mg/kg, respectively. Drug administration began 2 days before endothelial injury was induced and was continued until the animals were killed. Control animals received an equal volume of 0.5% CMC.

Induction of a neointima

Intimal thickening in the femoral artery of rats was produced by using photochemically induced endothelial injury as described previously (16,22). This model represents a nonmechanical method of producing vessel-wall denudation. In brief, animals were anesthetized by intraperitoneal injection of 50 mg/kg sodium pentobarbital, and a cannula was inserted into the jugular vein for rose bengal administration. The left femoral artery in the rat was carefully exposed, and a pulse-Doppler probe (PVD-20; Crystal Biotech, Hopkinton, MA, U.S.A.) was attached to it for monitoring blood flow. Transillumination with green light (wavelength, 540 nm) was achieved by using a xenon light with both a heat-absorbing filter and a green filter (L4887; Hamamatsu Photonics, Hamamatsu, Japan). Irradiation was directed via an optic fiber positioned 5 mm away from a segment of intact femoral artery proximal to the flow probe. Irradiation, at a dose of 0.8 W/cm^2, was initiated when the baseline blood flow was stable. Rose bengal (20 mg/kg) was then injected, and irradiation was continued for another 20 min. The femoral artery was considered to be occluded when the blood flow had completely stopped. The time required to occlude the artery after administration of rose bengal was considered as the occlusion time. Thirty minutes after rose bengal administration, the wound was closed.

Assessment of intimal thickening

The femoral artery was removed from each rat for histopathologic examination 21 days after endothelial injury. Vessels were perfusion-fixed in situ at physiologic pressure (80–100 mm Hg) with 1% paraformaldehyde and 2% glutaraldehyde in 0.1 *M* phosphate-buffered saline at pH 7.4. Then the femoral artery was removed and fixed further by overnight immersion in the same fixative. The specimens were sectioned transversely and stained with hematoxylin and eosin for light microscopy. The cross-sectional areas of the intima and media were calculated by using a computerized apparatus (Video Micro Meter Model VM-30; Olympus, Tokyo, Japan).

Cell culture

Rat A10 vascular smooth-muscle cells (A10 cells) were obtained from Dainippon Pharmaceutical Co., Ltd. (Osaka, Japan) and were maintained in Dulbecco's modified Eagle's medium containing 10% fetal bovine serum (FBS), 100 unit/ml penicillin, 100 μg/ml streptomycin, and 100 μg/ml kanamycin. The cells were incubated at 37°C in a humidified atmosphere containing 5% CO_2 in air.

Measurement of bromodeoxyuridine incorporation into cells

The effects of pemirolast and tranilast on DNA synthesis were determined by measuring 5-bromo-2'-deoxy-uridine (BrdU) incorporation. A10 cells (4 × 10^4 cells/well) were plated into 96-well plates in the medium supplemented with FBS and grown to confluence (4 days). They were then made quiescent by a 2-day incubation in FBS-starved medium containing 0.1% bovine serum albumin (BSA). The FBS-starved medium containing 10 ng/ml recombinant human platelet-derived growth factor (PDGF)-BB was added to the cells together with pemirolast or tranilast and BrdU (10 μ*M*). After a 24-h incubation period, the incorporation of BrdU into DNA was measured by using an enzyme immunoassay kit (Boehringer Mannheim GmbH Biochemica, Mannheim, Germany).

Measurement of cell migration

The migration of A10 cells was assayed by the modified Boyden chamber method by using a microchemotaxis chamber and a polycarbonate membrane filter with pores of 8 μm in diameter (Nucleopore, Pleasanton, CA, U.S.A.). The drugs were placed in the upper and lower chambers, and 10 ng/ml PDGF was placed in the lower chamber. A10 cells (10^5 cells/well) in FBS-starved medium were loaded into the upper chamber and incubated for 4 h at 37°C in a humidified atmosphere containing 5% CO_2 in air. The filter was fixed and stained with Diff-quik (Green Cross, Osaka, Japan). The number of A10 cells in four fields (0.25 mm^2) that migrated to the lower surface of the filter was determined by using a microscope.

Drugs

Pemirolast and tranilast were gifts from Tokyo Tanabe Co., Ltd. (Tokyo, Japan) and Kissei Pharmaceutical Company (Matsumoto, Japan), respectively. PDGF was purchased from Funakoshi Company (Tokyo, Japan). FBS was from GIBCO (Grand Island, NY, U.S.A.).

Statistical analyses

Results represented the mean ± SEM values. Statistical analysis was performed by analysis of variance (ANOVA) followed by Dunnett's multiple comparison test. A p value < 0.05 is considered to be significant.

RESULTS

Effect of pemirolast on intimal thickening

Twenty-one days after endothelial injury, neointimal thickening was observed in the subendothelial layers of the drug-untreated animals (Fig. 1A). Pemirolast at doses of 0.1, 1, and 10 mg/kg/day and tranilast at a dose of 300 mg/kg/day were orally administered 2 days before endothelial injury, and administration continued until the animals were killed. Intimal thickening tended to be reduced in animals receiving pemirolast at a dose of 0.1 mg/kg and tranilast at a dose of 300 mg/kg compared with controls (Fig. 1B, E). With pemirolast (1 and 10 mg/kg, Fig. 1C, D), intimal thickening was distinctly decreased compared with the control group (Fig. 1A). The values obtained from cross-sectional areas of the intima in the injured femoral arteries are shown in Fig. 2. The intimal areas in the pemirolast-treated groups (0.1, 1, and 10 mg/kg) were 2.10 ± 0.33, 1.36 ± 0.19, and 1.35 ± 0.18 (× 0.01 mm^2), respectively; values for the pemirolast-treated groups receiving dosages of 1 and 10 mg/kg were significantly ($p < 0.01$) decreased compared with the value for the control group (2.83 ± 0.49 × 0.01 mm^2, Fig. 2A). The ratios of the intimal area to medial area in the pemirolast-treated groups were 0.43 ± 0.07, 0.27 ± 0.04, and 0.26 ± 0.04, respectively; the last two values were

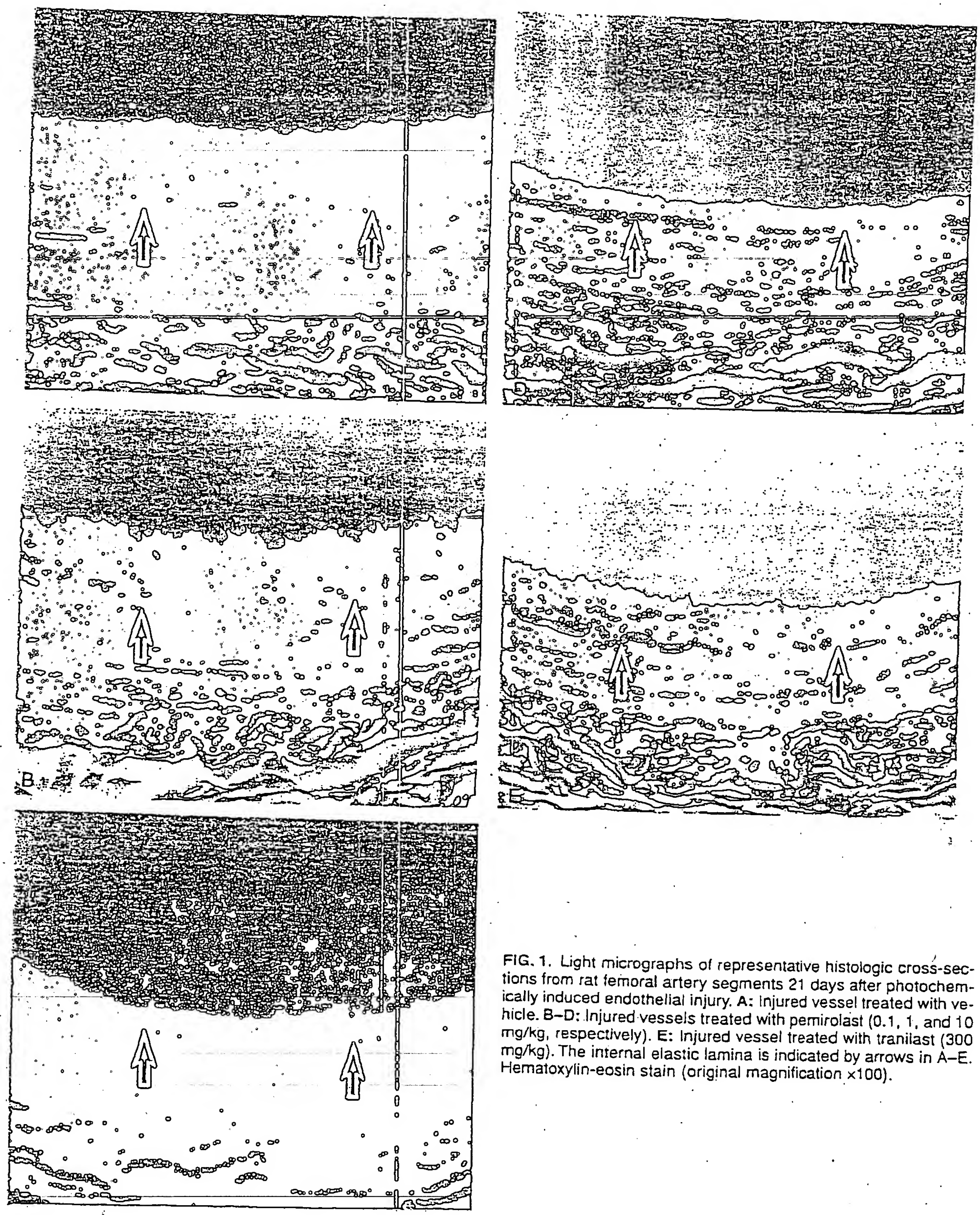

FIG. 1. Light micrographs of representative histologic cross-sections from rat femoral artery segments 21 days after photochemically induced endothelial injury. **A:** Injured vessel treated with vehicle. **B–D:** Injured vessels treated with pemirolast (0.1, 1, and 10 mg/kg, respectively). **E:** Injured vessel treated with tranilast (300 mg/kg). The internal elastic lamina is indicated by arrows in A–E. Hematoxylin-eosin stain (original magnification ×100).

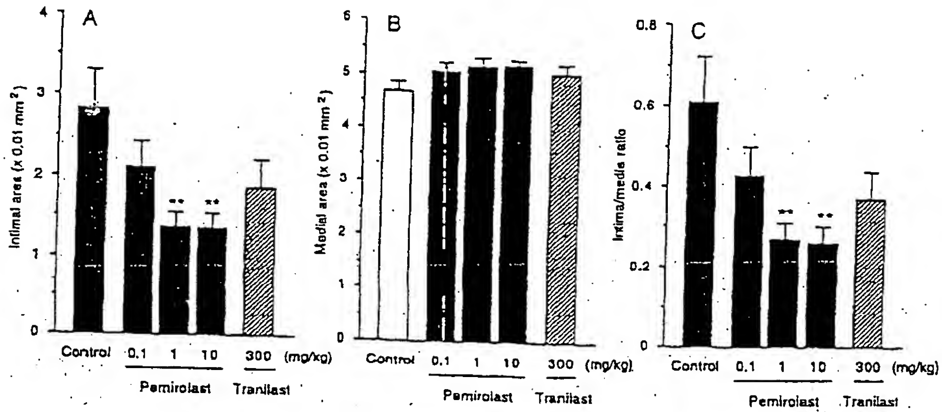

FIG. 2. Effects of pemirolast and tranilast on (A) intimal area, (B) medial area, and (C) intima/media ratio of rat femoral artery 21 days after photochemically induced endothelial injury. Data are presented as mean ± SEM (n = 15). **$p < 0.01$ versus values obtained from vehicle-treated rats.

significantly ($p < 0.01$) lower than that in the control group (0.61 ± 0.11; Fig. 2C). In the tranilast-treated group (300 mg/kg), the intimal area and the ratios of intimal area to medial area were 1.86 ± 0.35 (× 0.01 mm^2) and 0.37 ± 0.07, respectively; these values did not reach statistical significance compared with controls (Fig. 2A, C). The medial areas of pemirolast- and tranilast-treated groups were almost the same as that of the control group, and these values did not differ significantly from the control value (Fig. 2B). Neither drug had an effect on the medial areas in the arteries on the opposite side (uninjured arteries).

Pretreatment with pemirolast and tranilast had no effect on the occlusion time of the injured femoral artery, the values being 6.58 ± 0.90 min for the control group and 7.08 ± 0.92, 5.79 ± 0.50, and 6.87 ± 0.84 min for the pemirolast (0.1, 1, 10 mg/kg) and 5.62 ± 1.03 for tranilast (300 mg/kg) groups.

Effects of pemirolast and tranilast on A10 cell proliferation

The effects of pemirolast and tranilast on DNA synthesis were evaluated by measuring BrdU incorporation after a 24-h incubation period (Fig. 3). Pemirolast and tranilast produced a concentration-dependent inhibition of A10 cell proliferation stimulated by PDGF. Pemirolast and tranilast inhibited BrdU incorporation at concentrations of > 3 and 30 μM, respectively.

Effects of pemirolast and tranilast on A10 cell migration

The effects of pemirolast and tranilast on A10 cell migration stimulated by PDGF after a 4-h incubation period are shown in Fig. 4. Pemirolast and tranilast inhibited A10 cell migration stimulated by PDGF in a concentration-dependent manner. Pemirolast and tranilast inhibited cell migration at concentrations of > 3 and 30 μM, respectively.

DISCUSSION

In this study, we used the photochemical-induced thrombosis model in the rat femoral artery. This model involves an occlusion induced by a photochemical reaction between green-light irradiation and rose bengal, a photosensitizing dye, and can be applied to various animals, including mice, rats (16,23–25), and guinea pigs (22), and various sites including the femoral artery (16,22), middle cerebral artery (23), inner ear microcirculation (24), and coronary artery (25). Photoexcitation of rose bengal by green light–generated singlet oxygen causes endothelial injury followed by platelet adhesion, aggregation, and the formation of a platelet-rich thrombus at the site of the photochemical reaction (26). In this model, growth factors (including PDGF and other mitogenic factors, epidermal growth factor, and β-transforming growth factor) are released from adherent platelets and injured endothelial cells; these factors may play a key role in neointimal accumulation.

Pemirolast has a more potent antiallergic action than does tranilast in its inhibitory effects on passive cutaneous anaphylaxis and histamine release from mast cells or lung fragments (20,21). Its main mechanism may be the inhi-

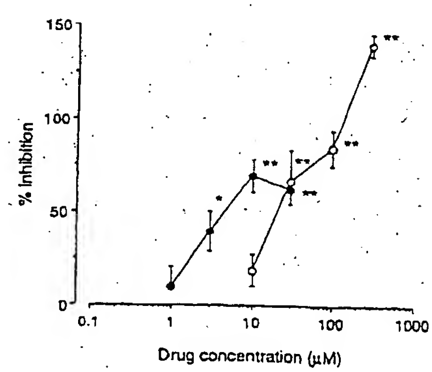

FIG. 3. Effects of pemirolast (solid circle) and tranilast (open circle) on the incorporation of 5-bromo-2'-deoxy-uridine (BrdU) into A10 cells stimulated by platelet-derived growth factor (PDGF). Growth-arrested A10 cells were incubated for 24 h together with PDGF (10 ng/ml) and BrdU (10 μM) in the presence of pemirolast or tranilast. Each value shows the percentage inhibition of BrdU incorporation into A10 cells when the control value is 100%. Data are presented as mean ± SEM (n = 6). **$p < 0.01$ versus values obtained from only PDGF-stimulated cells.

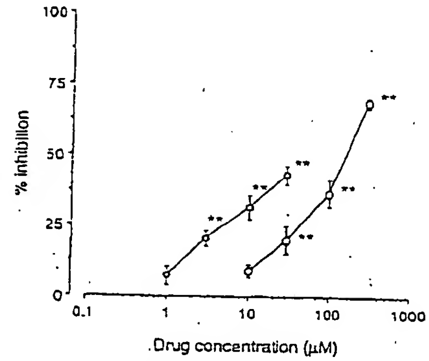

FIG. 4. Effects of pemirolast (solid circle) and tranilast (open circle) on the migration of A10 cells stimulated by platelet-derived growth factor (PDGF). Drugs were placed in the upper and lower chambers, and the lower chamber contained PDGF (10 ng/ml). A10 cell suspension was incubated for 4 h in the upper chamber. Each value shows the percentage inhibition of migrated cells when the control value is 100%. Results are presented as mean ± SEM (n = 5). **$p < 0.01$ versus values obtained from only PDGF-stimulated cells.

bition of the activation of phospholipase C and the decrease in signal-transducing molecule formation for mast cell degranulation (27,28). We previously reported that tranilast, at a dose of 300 mg/kg, significantly inhibited the formation of neointima by using the same model in spontaneously hypertensive rats (SHRs) (16). Therefore in this study to verify whether or not antiallergic agents inhibit intimal thickening, we investigated the effect of pemirolast on intimal thickening after endothelial injury and compared results with those of tranilast. We used normotensive rats in this study, because to investigate the direct effect(s) of antiallergic agents on intimal thickening, the possible influence of hypertension should be avoided. Sufficient intimal thickening was induced in this study by regulating the amount of administered rose bengal and intensity of green-light irradiation. Intimal thickening in this experimental condition was reduced when tranilast was administered at a dose of 300 mg/kg in comparison with findings for controls, but the values did not reach statistical significance. The different results may be explained by the more severe injury to the endothelium in this study compared with that in the previous experiment. In fact, the value obtained for the intima/media ratio in this study was 0.61 ± 0.11, which was distinctly greater than that of intima/media in the previous experiment, 0.17 ± 0.03. Under this condition, pemirolast remarkably inhibited intimal thickening at a dose of only 1 mg/kg after endothelial injury. Pretreatment with pemirolast or tranilast had no effect on thrombotic occlusion time of the femoral artery in this study, and it has been reported that the two compounds do not inhibit platelet aggregation in vitro (16,29). Therefore pemirolast and tranilast did not affect platelet function in this study.

In this study, we demonstrated that in rat A10 vascular smooth-muscle cells, pemirolast and tranilast suppressed the migration and proliferation of smooth-muscle cells stimulated by PDGF, and that pemirolast exhibited a more potent inhibitory effect than did tranilast. Pemirolast inhibited both proliferation and migration at a concentration of 3 μM, which is comparable to the maximal plasma concentration of pemirolast when pemirolast was orally administered in rats at a dose of 1 mg/kg (data not shown). Vascular smooth-muscle cell migration from the media to the intima and proliferation of smooth-muscle cells in the intima play key roles in intimal thickening. These results suggest that antiallergic agents directly inhibit the migration of smooth-muscle cells to the intima from the media and cellular proliferation in the intima. Further, the inhibitory effects of pemirolast on hyperplasia, as well as antiallergic effects, were observed at a lower dose compared with tranilast; thus antiallergic action(s) are considered to be related to antihyperplasia. Recently it was reported that pemirolast inhibited histamine release from mast cells via inhibition of FcεRI [receptor immunoglobulin E (IgE)]-mediated phospholipase C activation, followed by suppression of the production of inositol 1,4,5-trisphosphate (IP_3) and 1,2-diacylglycerol (1,2-DG) by hydrolysis of phosphatidylinositol 4,5-bisphosphate (27,28). IP_3 mobilizes Ca^{2+} efflux from intracellular Ca^{2+} storage site(s), and 1,2-DG activates protein kinase C. These events stimulate degranulation in mast cells. Smooth-muscle cell proliferation stimulated by PDGF is related to activation of intracellular signal transduction via PDGF receptor(s), and phospholipase C activation also is considered to be essential for cell proliferation stimulated by PDGF in these signal-transducing molecules (30). In fact, it has been reported that two selective protein kinase C inhibitors inhibited smooth-muscle cell proliferation (31). The intracellular signal-transducing molecule(s) for histamine release from mast cells may be the same as those for smooth-muscle cell proliferation, and antiallergic agents may suppress both pathways. Further study is necessary to clarify the detailed mechanism(s) through which antiallergic agents suppress hyperplasia after endothelial injury. A recent report indicated that antiallergic agents reduced the rate of restenosis after PTCA in humans. Pemirolast (32) at a dose of 20 mg/day reduced the restenosis rate after PTCA in comparison with controls (17.4 vs. 40.0%), as did tranilast (17,18) at a dose of 600 mg/day (14.7 vs. 46.5%). Antiallergic agents have a broad spectrum of pharmacologic actions, including the inhibition of cytokine and leukotriene release from leukocytes (33,34) and the inhibition of phosphodiesterase (21) and phospholipase A2 (27) activities. Thus, multiple actions of antiallergic agents may be related to the prevention of restenosis after PTCA in humans.

Based on our observations, antiallergic agents may prove to be valuable agents for the prevention of atherosclerosis and restenosis after PTCA. Pemirolast has more potent antihyperplastic action than does tranilast.

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